Mitochondrial Genome Assemblies of *Elysia timida* and *Elysia cornigera* and the Response of Mitochondrion-Associated Metabolism during Starvation

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Abstract

Some sacoglossan sea slugs sequester functional plastids (kleptoplasts) from their food, which continue to fix CO_2 in a light dependent manner inside the animals. In plants and algae, plastid and mitochondrial metabolism are linked in ways that reach beyond the provision of energy-rich carbon compounds through photosynthesis, but how slug mitochondria respond to starvation or alterations in plastid biochemistry has not been explored. We assembled the mitochondrial genomes of the plastid-sequestering sea slugs *Elysia timida* and *Elysia cornigera* from RNA-Seq data that was complemented with standard sequencing of mitochondrial DNA through primer walking. Our data confirm the sister species relationship of the two Sacoglossa and from the analysis of changes in mitochondrial-associated metabolism during starvation we speculate that kleptoplasts might aid in the rerouting or recycling of reducing power independent of, yet maybe improved by, photosynthesis.

Key words: photosynthetic slugs, mitochondrial genomes, energy metabolism, starvation, ROS stress.

Introduction

Sacoglossan sea slugs, with very few exceptions, feed on siphonaceous algae by piercing their cell walls and sucking out the cytosolic content. Most species can specifically sequester the plastids, known as kleptoplasts, from the nutriment mix (Trench 1969). Of the roughly 400 Sacoglossa species that are described (Jensen 2007; Martin and Wägele 2014), about 75 species are able to retain kleptoplasts that continue to fix CO_2 in a light-dependent manner in the cytosol of cells that form the digestive tract (Trench et al. 1973, 1974; Hinde 1978; Christa, de Vries, 2014; Christa, Zimorski, et al. 2014; de Vries et al. 2015). How the kleptoplasts stay active remains a key question, as it occurs in the absence of algal nuclear support (Wägele et al. 2011; Bhattacharya et al. 2013; Rauch et al. 2015) commonly thought to be essential.

The motive behind kleptoplasty in sacoglossan sea slugs remains elusive and the importance of on-going photosynthesis and CO_2 fixation is under debate (Christa, Zimorski, et al. 2014; Pierce et al. 2015; de Vries et al. 2015; Laetz et al. 2017). It remains uncertain to what degree CO_2 fixation contributes to the overall energy budget of the slugs and alternative reasons are rarely considered (Christa, de Vries, et al. 2014; de Vries et al. 2014). A comparison of the sister species *Elysia timida* and *Elysia cornigera*, which both feed on *Acetabularia acetabulum*, demonstrated that *E. cornigera* dies in the presence of CO_2 fixing kleptoplasts, and therefore in the presence of accumulated ROS, while *E. timida* endured starvation possibly through the suppression of reactive oxygen species (ROS) (de Vries et al. 2015).

Investigating kleptoplasty in Sacoglossa usually focuses on the performance of the kleptoplasts. From work on plants it is known that plastid and mitochondrial function are connected (Hoefnagel et al. 1998), but the effect of kleptoplasts and food deprivation on sea slug mitochondria has not yet been

explored. Here, we present the mitochondrial genomes of two sister species of Sacoglossa and a set of nuclear-encoded, mitochondrion targeted proteins whose expression changes we monitored simultaneously under different conditions. Our data seek to offer new resources and a new angle from which to study how kleptoplasts are being kept active by photosynthetic sea slugs.

Materials and Methods

Cultivation and Microscopy

Elysia timida was collected on Giglio (Italy; 42°22′ N, 10°52′ E and 42°21′ N, 10°52′ E) between 3 and 6 m depth and *Elysia cornigera* was collected on Spanish Harbor Key (Florida Keys, USA 24°38′ N, 81°18′ W) at up to 1m depth. Both *E. timida* and *E. cornigera* were reared at 21 °C under a 12hL:12hD rhythm at 25 µmol quanta m^{-2} s^{-1} in artificial sea water (ASW; 3.7% salinity, Tropic Marine) including water change every other day. For imaging, 1 week starved specimens of *E. timida* and *E. cornigera* were stained for 45 min with 2 µM MitoTracker Red CMXRos (excitation/emission HeNe 543/599 nm; LifeTechnologies) in 3.7% ASW, rinsed twice with ASW and decapitated. Confocal laser scanning microscopy was carried out with a Zeiss LSM 710. Images were processed with Fiji/ImageJ 1.48f (Schindelin et al. 2012).

Mitochondrial Genome Assembly

Mitochondrial (mt) genomes were primarily assembled from RNA-Seq data (de Vries et al. 2015) using Sequencer (Sequencher v. 5.3, Gene Codes Corporation, USA) and standard assembly settings. In addition, continuous stretches of ~6.5 kb of *E. timida* and ~12 kb of *E. cornigera* mitochondrial DNA were sequenced by primer walking to close gaps and compare RNA and DNA sequences. Genomic DNA was extracted with Plant DNAzol (ThermoFisher) and Phusion High-Fidelity DNA polymerase (New England Biolabs) used for standard PCR reactions. Amplification products were sequenced and fed into the Sequencher assembly. The sequences were found to be close to identical—but to 100% in terms of contiguity—to those of the sequenced RNA, with only occasional differences in base calls (about 3 per 1kbp), but no larger gaps indicating potential introns. Due to the nature of the samples it is not possible to distinguish between, for example, single-nucleotide polymorphisms or RNA editing, but if the latter is occurring at all, frequencies would be marginal. The average sequence coverage was 508,168 and 210,540 for *E. timida* and *E. cornigera*, respectively. Gene annotation was performed using MITOS (Bernt et al. 2013) and Geneious 8.0.3 (Biomatters, New Zeeland, Kearse et al. 2012) with the mt genomes of *Elysia chlorotica* (Rumpho et al. 2008), *Thuridilla gracilis* (Medina et al. 2011), and *Placida* sp. (Fan 2013) as references. Mitochondrial genome maps were generated using Organellar GenomeDRAW (Lohse et al. 2013).

Phylogenomics

For every protein-coding gene of the mitochondria we performed individual amino acid sequence alignments with Geneious 8.0.3 (Biomatters, New Zeeland, Kearse et al. 2012) and using *Siphonaria pectinata* (AY345049) as the outgroup. Alignments were performed using Fast Fourier Transform (MAFFT; Katoh and Standley 2013) with the G-INSI mode, inspected by Aliscore (Misof and Misof 2009) and conspicuous sites removed. All individual alignments were concatenated and a phylogenetic reconstruction performed using RaxML (Stamatakis 2006) with the LG + I+G + F model (four discrete gamma categories and sites), as suggest by ProtTest analyses (Abascal et al. 2005), and 1,000 bootstrap replicates.

Metabolic Pathway Mapping

Data of expressed genes involved in the mitochondrial metabolism of both *E. timida* and *E. cornigera* were extracted from a previous study (de Vries et al. 2015), based on their KEGG annotations (Ogata et al. 1999). In cases where KEGG IDs (e.g., K02262 representing COX3) represented multiple unigene IDs (Eti019163, Eti001642, and Eti000008), the sum of the expression values (in FPKM) were taken and fold changes calculated by log_2 of the FPKM expression value divided by the control (the average of triplicates). Hence, the unigene ID with the highest expression has the most influence on the average expression.

Results and Discussion

The Mitochondrial Genomes of *E. cornigera* and *E. timida* and Phylogenomic Analysis

We assembled the mitochondrial (mt) genomes of *E. cornigera* and *E. timida* based largely on transcriptome data. For both species, three gaps of about 50 bp, 500 bp, and 1 kbp were closed by PCR and Sanger sequencing, but in principle it demonstrates the expression of almost the entire mt genomes, which is common for genomes of both plastids and mitochondria (Smith and Lima 2016; Tian and Smith 2016). We obtained 14,118 and 14,088 bp of assembled, circular mt genomic sequences for *E. cornigera* and *E. timida*, respectively (fig. 1a). The two fully assembled slug mt genomes fit well within the conserved nature of the mt genomes of all animals (Boore 1999): both contain two ribosomal RNAs, 22 transfer RNAs and 13 protein-encoding genes (see supplementary table 1, Supplementary Material online). The mt genomes of the slugs are syntenic (fig. 1a) and match the general pattern of mt genome arrangement among other Sacoglossa (Medina et al. 2011; Greve et al. 2017). On a nucleotide level, the mt genomes of *E. cornigera* and *E. timida* have a pairwise identity of 86.2% and *E. timida* a pairwise identity of 71.1% to *Elysia ornata*, which brachnches basal to
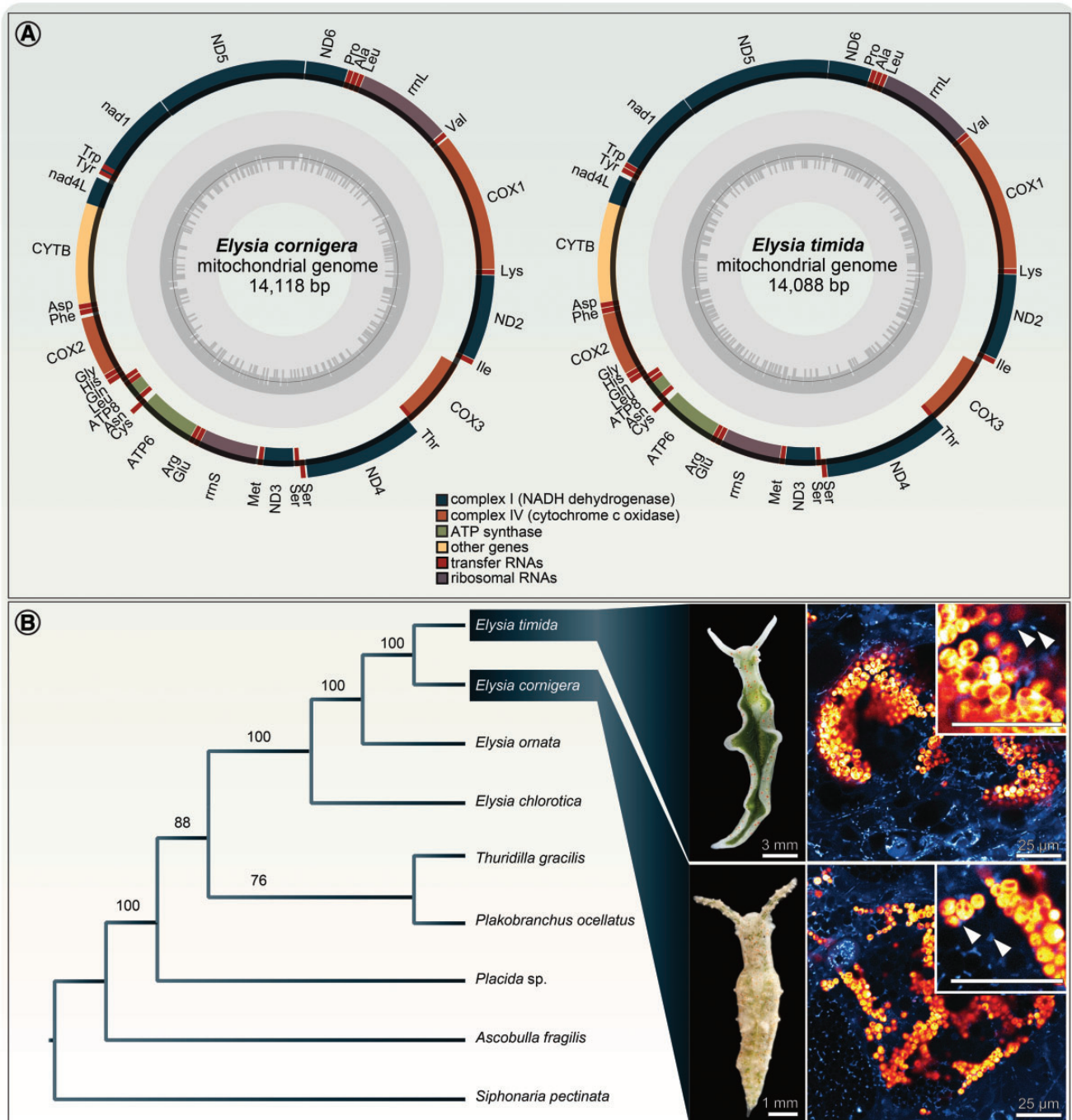
Fig. 1.—Mitochondrial genomes and sacoglossan phylogeny. (*a*) Maps of the two circular and syntenic mitochondrial genomes of *Elysia cornigera* and *Elysia timida* (accession numbers KU174945 and KU174946, respectively). The grey inner circles show the GC skew and the dark grey line marks the 50% GC threshold. (*b*) Phylogenetic tree of eight Sacoglossa and the false limpet *Siphonaria pectinate* as the outgroup, which corroborates the sister species relationship of *E. cornigera* and *E. timida*. Numbers indicate bootstrap values. The right panel shows of freshly fed slugs and confocal laser scanning micrographs of digestive tubules in which the kleptoplasts (false-coloured red-hot) are sequestered. Top right boxes are blow ups of digestive tubules with arrowheads pointing at mitochondria (blue) that often reside in close proximity to the kleptoplasts.

the two congener species in the phylogenetic tree (fig. 1*b*). Based on the 13 mt protein-coding genes and including all available sequences of complete Sacoglossa mt genomes, we performed a phylogenetic analysis. This supports the sister taxon relationship of *E. timida* and *E. cornigera* (fig. 1*b*) and is in line with a previous study (Christa et al. 2015). Our phylogenetic data furthermore supports the observed relationship of *Plakobranchus* and *Thuridilla* as a sister group to *Elysia* sp.

(Greve et al. 2017), which stands in contrast to an earlier study also based on mt genome analysis (Fan 2013).

Regulation of the Main Energy Pathways during Starvation

To study the effects of starvation on the core energy metabolism of plastid-housing slugs, we made use of global gene expression profiles of *E. timida* and *E. cornigera* specimens that had been exposed to three different conditions: starvation alone (S), starvation with the addition of the photosynthesis inhibitor drug monolinuron (S + M; cf. Arrhenius et al. 2004), and starvation with a 1-h daily high light treatment (S + B) with 1,000 µmol m^{-2} s^{-1}. From this data set, we extracted major pathways associated with mitochondrial physiology and metabolism such as the TCA cycle, the glycolytic pathway and oxidative phosphorylation pathway (OXPHOS). For the analysis only those genes were taken into account that were upregulated (log$_2$[FC$_{condition/t0}$]≥1) in comparison to the unstressed, fed state of the slugs. The trend for the gene regulation of the pentose phosphate pathway, the glycolysis pathway, the fatty acid pathway, and the tricarboxy acid cycle (TCA) all showed a similar pattern for both species and among the three different starvation treatments. In general, in *E. timida* the upregulating of these pathways tended to be more pronounced during the first days of starvation, while during prolonged starvation this upregulation ceased (supplementary fig. 1, Supplementary Material online).

High Light Stress Alters the Expression of the OXPHOS Pathway in *E. timida*

When *E. timida* is deprived of its food source, 35% of the proteins of the OXPHOS machinery are up-regulated after 4 days of starvation (fig. 2). For the up-regulation of the OXPHOS pathway to be functional, it requires a constant influx of reducing equivalents. Simultaneously, however, we observed a downregulation of the TCA cycle. This raises the question of the source of alternative reducing equivalents in a starving and plastid-housing slug. In many eukaryotes, reducing equivalents can be imported via the malate-aspartate and glycerol phosphate shuttle (Eto et al. 1999). In the slugs, most genes coding for proteins involved in these shuttles were downregulated after 4 days of starvation (dos) (supplementary table 2, Supplementary Material online), but it remains a possibility that the import of additional reducing equivalents from functional kleptoplasts further delays the shutdown of the OXPHOS pathway, like it occurs in plant cells (Hoefnagel et al. 1998). The latter is important, because any kind of OXPHOS pathway destabilization fosters the generation of ROS (Zorov et al. 2014).

Blocking of photosynthesis through cadmium (Cd) in *Arabidopsis* leads to an outburst of ROS (Bi et al. 2009). Like the Cd treatment, the treatment with monolinuron manipulates the flow of the electron-transport-chain (ETC), likely leading to the generation of problematic ROS levels.

The latter could trigger the upregulation of the OXPHOS pathway of the mitochondria, which has been described to curb ROS-induced damage (Tanaka and Hanaoka 2013). In *E. cornigera*, the increased regulation of the OXPHOS pathway is maintained when the kleptoplasts are high light-stressed (23% and 27% after 4 and 7 dos, respectively; fig. 2b) and *E. timida* sustains a high level of regulation for the first week of starvation that ceases at the end of the starving period (78% and 81% after 4 and 7 dos, respectively, and 10% after 30 dos; fig. 2b).

When the kleptoplasts experience abiotic stress such as high light or a interference of photosynthesis function through monolinuron, it likely increases H$_2$O$_2$ levels like it does in plant cells, in which it is known that high light-derived ROS is a major hub in stress signalling (Miller et al. 2010; Apel and Hirt 2004; Shapiguzov et al. 2012). H$_2$O$_2$ is also indirectly generated through the ETC of the mitochondrial OXPHOS pathway (Murphy 2009) and perceived as a signal by the cell (Lee et al. 2011). It might be that the epithelial cells which harbour kleptoplasts cannot distinguish between H$_2$O$_2$ originated from the stolen organelle and its own mitochondria in the cell the same way plants can (Sewelam et al. 2014); they might solely receive it as a sign of a damaged mitochondria ETC. As a result, the turnover rate of the main ETC enzymes complexes is increased (Sharma et al. 2012), which in *E. timida* results in less ROS production from the mitochondria, because damaged ETC components are being replaced faster (D'Autreaux and Toledano 2007). The cell might register the ROS released by the kleptoplasts as retrograde signals from the mitochondria (Plecitá-Hlavatá and Ježek 2016). The ROS of two sources might booster the repair of one critical pathway through mechanisms common to eukaryotic cells (Sharma et al. 2012; Choudhury et al. 2016).

Conclusion

Research on sacoglossan sea slugs has focused to a large degree on the photosynthetic capacity of the sequestered kleptoplasts and their potential contribution to tolerate starvation through providing a source of energy-rich carbon compounds. Starvation tolerance in Sacoglossa, however, is evidently more complex and other physiological processes should be considered. Our mitochondrial genome data confirm the phylogenetic relationship of the two Sacoglossa, whose comparative analysis has proven useful in the past (de Vries et al. 2015). Based upon that data, our mitochondrion-focused screen for gene expression changes suggests that starving Sacoglossa uphold and even increase the expression of genes encoding for the OXPHOS pathway. Here, kleptoplasts might come into play by providing reducing equivalents for the animal's mitochondria, herby supporting ongoing ATP production and furthermore suppressing ETC-induced ROS stress. ROS production is likely also higher due to the presence of photosynthesising kleptoplasts, which might
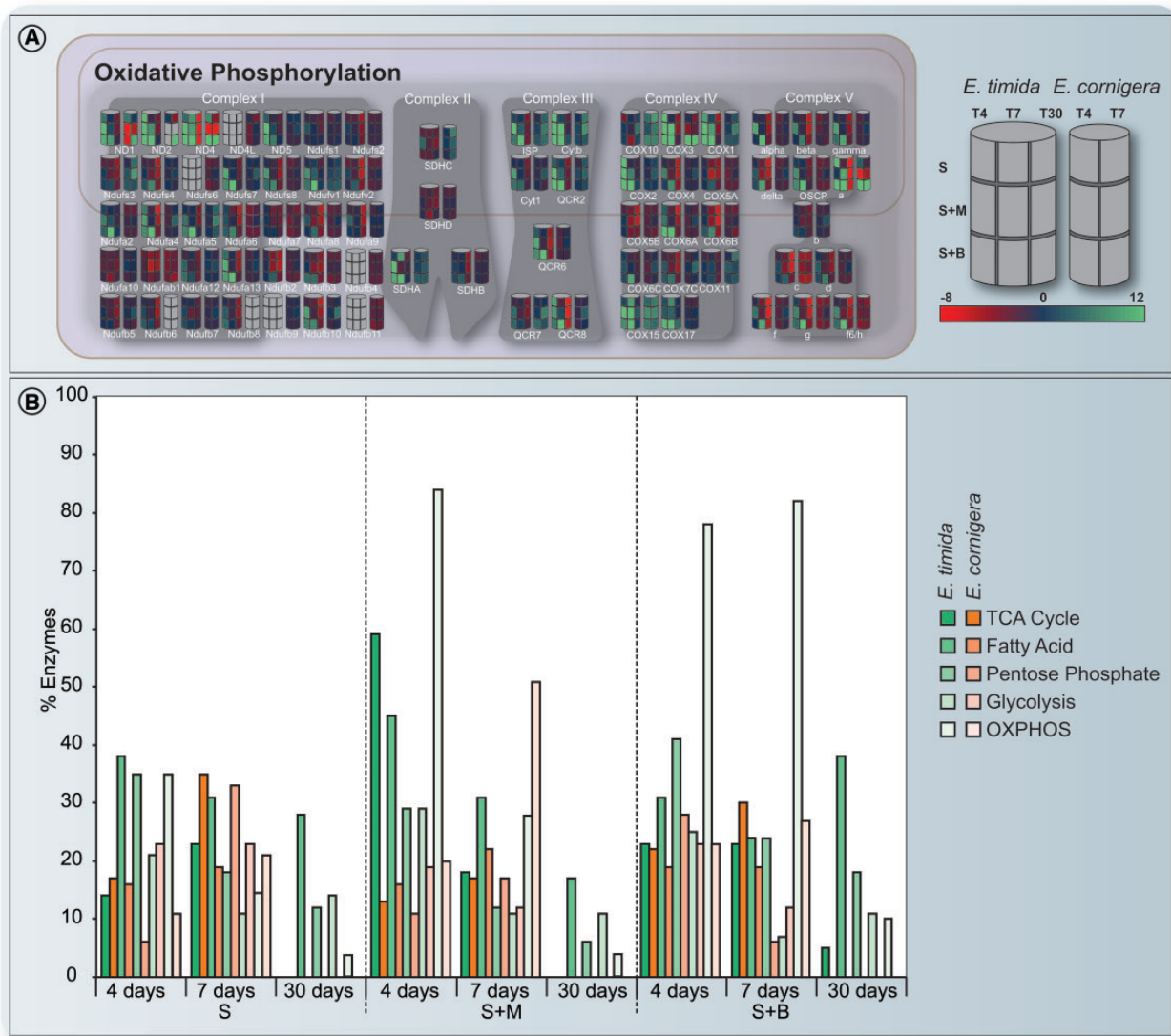
Fig. 2.—Gene expression changes in the OXPHOS and other mitochondrion-related pathways. (*a*) Gene expression shifts of the OXPHOS metabolic pathway. KEGG-based map of oxidative phosphorylation. Coloured "barrels" displays gene expression levels for starving *E. cornigera* and *E. timida* starved for 4, 7, and 30 days (T4, T7, T30) relative to the freshly fed state; upregulation is shown from green to blue, downregulation from blue to red, and grey boxes indicate no detectable gene expression for the given condition. Each barrel represents an enzyme, with the wider barrels representing *E. timida* due the extra data point of day 30 (T30). Multiple barrels at one step in the metabolic pathway represent a protein complex (e.g., complex I); note that one enzyme (or complex) can act at multiple steps in the pathway and in different complex compositions. For every enzyme (complex) the two species are shown adjacent to each other for comparison. Relevant substrates are indicated by dots and labelled with their common name. Note some steps of the fatty acid pathway occur in the mitochondrion. (*b*) Frequency of upregulation among metabolic pathway-associated gene expression. Bardiagram of the percentage of upregulation (relative to T_0: $\log_2[FC_{condition/t0}] \geq 1$) of gene expression associated with the 5 main mitochondrial energy metabolism pathways; all enzymes of one pathway are added up to represent 100%. Note that only in the case of oxidative phosphorylation (OXPHOS) in *E. timida* (and with either monolinuron or bleaching treatment), 80–100% of the associated enzymes are observed to be upregulated. This might reflect a response of the animal to an increase of reactive oxygen species actually stemming mainly from abiotically stressed kleptoplasts and not the mitochondria themselves. For details please refer to the text. TCA, tricarboxy acid cycle; S, starvation alone; S + M, starvation with additional 2 µg/ml of the photosynthesis inhibitor drug monolinuron; S + B, starvation with a 1-h daily high light (1,000 µmol m^{-2} s^{-1}) treatment.

enhance the endogenous response in the animal's cells that respond to alleged mitochondrial stress. The latter possibility is supported by the similar, but enhanced response observed upon abiotic stress. Our results suggest that the integration of kleptoplasts is maybe more involved then so far considered. This encourages to further investigate to which degree the interplay of kleptoplasts and animal mitochondria are linked in ways that resemble those studied in algae and plant cells.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Acknowledgments

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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